EXHIBIT D

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Quantitative Single Cell Analysis and Sorting

Rapid analysis and sorting of cells is emerging as an important new technology in research and medicine.

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Direct quantitative measurement of biochemical and biophysical cellular properties of individual cells is becoming increasingly important in biomedical research and clinical medicine. This is evidenced by the fact that many pathological changes in cells may be recognized by measurable changes in constituent substances and not the presence or absence of a particular substance. For example, an increase in nuclear fluorescence from cells stained with Acridine Orange may be used as a description of malignant cells. The presence of an extra male chromosome, detectable by DNA analysis, is characteristic of patients with Klinefelter's syndrome. These abnormalities may only be detected by measurements made on a cell by cell basis.

Physical sorting of cells by type to achieve pure populations for study has become a necessity. Many separation methods rely on physical properties such as size or density or differences in surface membrane properties such as charge. While useful, these methods are limited by the restricted set of parameters upon which separation can be based and by the fact that they are batch techniques.

New flow cytometers and sorters permit quantitative multiparameter measurements of cellular properties at rates of several thousand cells per second and the physical sorting of selected subpopulations of cells based on these measurements (*1*, *2*). Most important, analysis and sorting are performed on a cell by cell basis. The range of cellular substances and properties now open to measurement and on which cell or cell component sorting may be based is limited only by the lack of a probe, the signal-to-noise ratio achievable during measurement, and our imagination. The high rates of cell analysis provide statistical precision and sensitivity. An understanding of the instrumentation is essential for full realization of the potential applications. In this article we discuss the instrumentation for this technology and describe some of the diverse applications of flow cytometers and sorters.

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Instrumentation

An electrical technique for cell counting and sizing was described by Coulter (*3*) in 1956. This became the basis for the first viable flow analyzer. Cells are counted and sized as they flow in a conductive liquid through a small orifice (75 to 100 μm) between two chambers.

A two-parameter flow cytometer that measured absorption and back-scattered illumination of unstained cells to determine cell nucleic acid content and size was reported on by Kamentsky (*4*) in 1965. While these measurements are seldom used today, this instrument represented the first multiparameter flow cytometer.

The first cell sorter was described by Fulwyler (*5*) that same year. By means of an electrostatic deflection ink-jet recording technique developed by Sweet (*6*), the instrument was capable of sorting cells based on their volume (Coulter principle) at 1000 cells per second. It was noted that, in theory, the system was capable of separating cells according to any electronically measurable characteristic.

Flow cytometers were improved on by Van Dilla *et al.* (*7*) in 1969 by incorporating a laminar sheath-flow technique and argon-ion laser. The sheath-flow technique, first described by Crosland-Taylor (*8*), confines cells to the center of the flow stream. Use of a laser provided enough power at the proper wavelengths to permit fluorescence measurements to be made. A helium-neon laser and the same flow chamber were used by Mullaney *et al.* (*9*) to study small-angle, forward light scattering of cells in flow. Scattered light between 0.5° and 2° was collected and provided distributions similar to those obtained by using a Coulter counter.

Other important innovations included a cell-sorting instrument by Hulett *et al.* (*10*) which performs measurements on cells in a liquid jet in air rather than in a flow chamber, and a flow analyzer by Dittrich and Göhde (*11*) achieving high sensitivity with a flow-chamber design incorporating epi-illumination.

The previous systems all utilized excitation beams and viewing resolutions larger than cells of interest in the flow stream. Such systems are termed zero resolution. Wheeless and Patten (*12*) developed a low-resolution slit-scan technique to provide limited morphological information on cells. The technique was adapted to a flow system making it possible to distinguish between nuclear and cytoplasmic fluorescence, and nuclear and cell size.

This brief history represents only the beginning of instrumentation development carried out in numerous laboratories which has led to the sophisticated and useful cell analysis and sorting instruments available today.

Cell Transport

Fluid transport of cells in suspension through a measurement region is fundamental to any flow analysis or sorting instrument. Most instruments utilize a laminar sheath-flow technique (*8*) to confine cells to the center of the flow stream and reduce clogging due to clumps. Cells enter the flow chamber under pressure through a specimen needle which is surrounded by sheath fluid, Fig. 1A. Sheath diameter is generally 50 to 100 μm for cell sorters and up to 250 μm for cell analyzers. Sheath and specimen pressures are adjusted to provide the desired specimen stream diameter, generally 10 to 30 μm. Cell concentrations are adjusted to achieve a sequential flow of primarily single cells, sufficiently separated so that only one cell is subject to measurement or sorting at a given time. Furthermore, overlapping cells can be detected and electronically eliminated from consideration.

Measurement

Flow cytometers are generally multiparameter, recording several measurements on each cell. Typical measurements include Coulter volume, fluorescence at one or more wavelengths, and low-angle forward light scatter (*13*). A laser source is used for most fluorescence and scatter measurements because of its intensity and monochromatic properties. Beam-shaping optics spread the laser excitation beam across the specimen stream and compress the beam in the direction of fluid flow to achieve desired beam thickness, typically 50 μm. Flow cytometers record measurements on cells within the flow chamber while in most sorters the coaxial specimen and sheath streams jet out into air before they intersect the laser beam.

Fluorescence is excited as cells traverse the laser excitation beam and the fluorescence is collected by optics placed 90° to the incident beam (Fig. 1A). A barrier filter blocks laser excitation illumination, while a dichroic mirror and filters are used to select bands of fluorescence for measurement. Photomultiplier tubes are typically used as detectors. Fluorescence measurements provide high sensitivity because of low background. A detection sensitivity of 3000 molecules of fluorescein per cell has been reported (*14*).

Objects in flow also scatter light as they intersect the laser beam. In most instruments the use of light scatter has been limited to small angle measurements for size determination or gating. A single detector (Fig. 1A) collects forward light scatter in the 0.5° to 2° range (*9*) (some instruments allow measurement up to 15°). This measurement, termed "near forward-angle scattering" produces a large signal dependent primarily upon volume of the cell under illumination.

Slit-Scanning

A low-resolution slit-scan technique is used to provide morphological information on cells as they pass through the excitation beam (Fig. 1B) (*15*, *16*). In a one-dimensional slit-scan flow system cells stained with a fluorochrome are sequentially illuminated as they flow through a line-focused laser beam, or "slit," (typically 5 μm thick) of fluorescence excitation illumination. Measurement of fluorescence from the cell at discrete time intervals as the cell passes through the excitation slit yields a one-dimensional fluorescence contour. Computer analysis of the contour yields cell diameter, nuclear diameter, nuclear-to-cell diameter ratio, cytoplasmic fluorescence, nuclear fluorescence, and certain other low-resolution morphological information such as information on binucleation or cell overlap.

Because slit-scan data analysis is more complex than zero-resolution data analysis, slit-scanning represents a compromise on throughput and resolution. Achievable resolution in flow is related to the dimension of the slit which, in turn, is related to the width of the specimen stream and size of cells to be scanned. Five-micrometer resolution is achievable for a 50-μm specimen stream, while 1-μm resolution demands a specimen stream of only 1.5 μm in diameter and would be practical only for slit-scanning of chromosomes and other small particles (*15*). The slit-scan technique has not yet been incorporated in a cell sorter.

Some morphological information is also available in conventional zero- and low-resolution flow systems. Pulse width analysis of the fluorescence or light-scatter signals may provide information on cell and nuclear diameters (*17–19*).

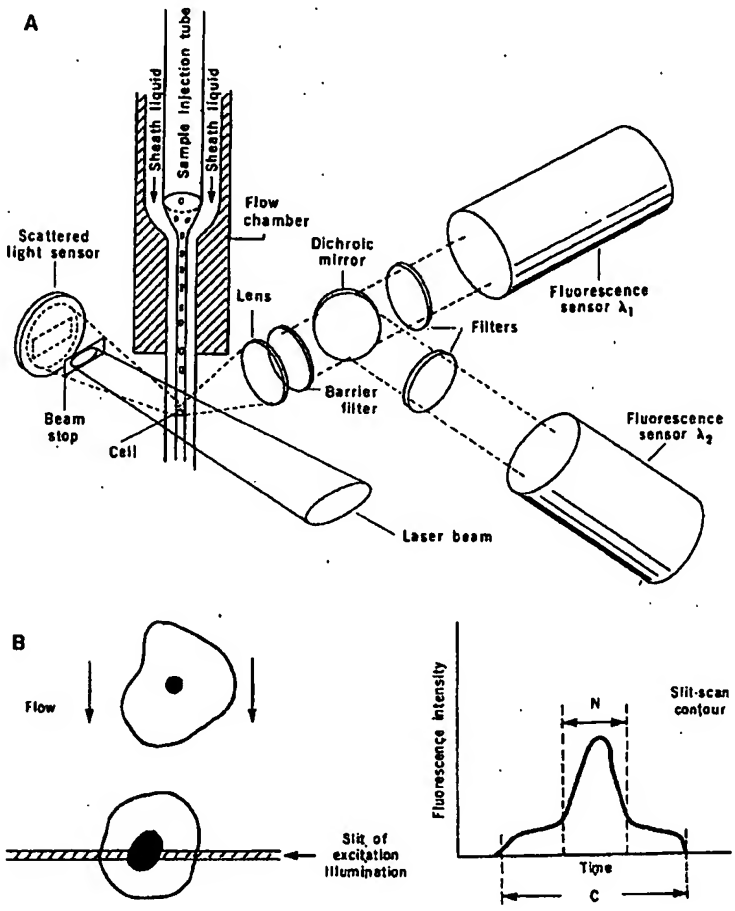

Fig. 1. (A) Generalized flow analyzer showing sample and sheath fluid paths, cell stream, laser illumination and light scatter, and fluorescence sensors. Sheath fluid flows coaxially around the sample injection tube and serves to center cells for measurement. Low-angle light scatter and fluorescence at two wavelengths are measured as cells traverse the laser excitation beam. (B) One dimensional slit-scanning. Cells are sequentially illuminated as they flow through a slit of laser excitation illumination. Measurement of fluorescence from the cell at discrete time intervals yields a fluorescence contour. Contours may be analyzed to provide cell diameter (*C*), nuclear diameter (*N*), nuclear to cell diameter ratio, nuclear fluorescence, cytoplasmic fluorescence, and certain other low-resolution morphological information.

Cell Sorting

Sorting is accomplished as cells exit from the flow chamber in a liquid jet (Fig. 1C). Vibrating the nozzle assembly at a high frequency (typically 30,000 hertz) by means of an ultrasonic transducer causes the stream to break up into a series of uniform droplets. Vibration at 30,000 hertz produces 30,000 droplets per second. Cells flowing through the instrument are isolated in these tiny droplets. By applying a charge to a droplet containing a cell of interest and passing the droplet stream through an electrostatic field, the charged droplets are deflected right or left, carrying the sorted cells.

Sort signals are derived from measurements recorded on cells in flow. Usually these measurements are made on cells in the liquid jet immediately after it leaves the flow chamber and before it breaks into droplets. The jet intersects one or more laser beams (*20*), and detectors record two-color fluorescence and light scatter from each cell. Signals produced are processed and delayed to produce the electrical pulses used to charge the liquid stream at the time when the droplet containing the desired cell is forming. Processing may be analog or digital (*21*). If two cells are so close together that they cannot be isolated, the sorting pulse is aborted thereby reducing coincidence effects. Cell sorting may be carried out under sterile conditions, and cell viability may be maintained during sort.

The instrumentation configuration providing measurements on cells in a liquid jet in air has advantages over instrumentation where measurements are recorded within a flow chamber: (i) it minimizes the time between measurement and droplet formation and this results in a purer sort; (ii) it permits higher sort rates; and (iii) it results in less dilution of the sorted specimen because fewer droplets need be charged per cell. Disadvantages include a more limited set of possible measurements and difficulties encountered in performing measurements on cells in a liquid stream in air.

Pulse Processing

Multichannel analyzers are used in data analysis to provide frequency distributions for each measured parameter or feature. Two-parameter correlated data are stored in some instruments but such data are usually limited to a 64 by 64 element data matrix. Sorting is based upon cells falling within preselected regions, or windows, of a particular feature distribution. Two windows are used for a two-direction sort, and features may be logically combined to create windows in multidimensional feature space. Cells may be selected for sort depending on whether they fall within or outside of a window. For example, "gated analysis" combines measurement of light scatter and fluorescence to form a useful window in two-dimensional feature space (*2*, *22*). The fluorescence frequency distribution shows overlap between cells and debris in the specimen whereas sorting reveals that particles having low light-scatter intensity are debris, those with midrange scatter intensity are primarily single cells, and those with high scatter intensities are primarily clumps. Only single cells are considered for fluorescence analysis and sorting by first requiring that the light-scatter signal intensity correspond to the midrange scatter intensity. Particles with high or low light-scatter intensity are not considered for fluorescence analysis.

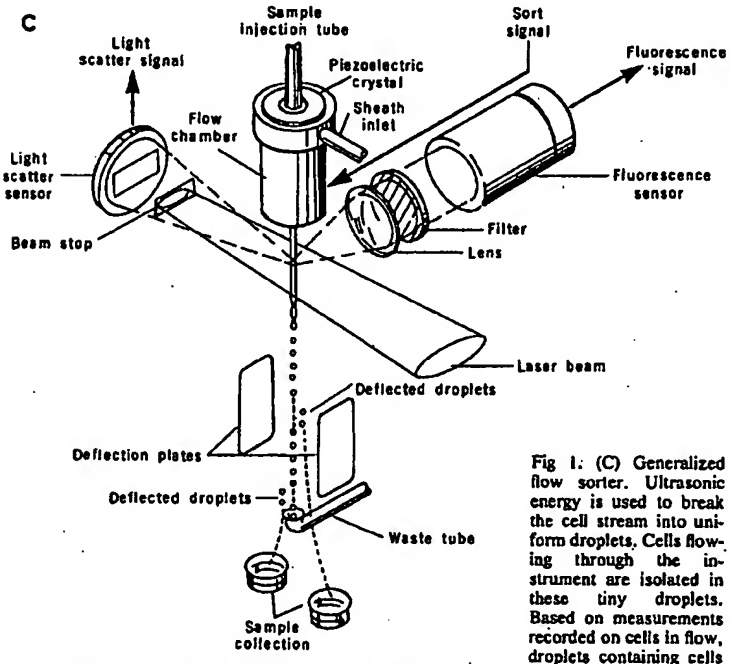

Fig 1: (C) Generalized flow sorter. Ultrasonic energy is used to break the cell stream into uniform droplets. Cells flowing through the instrument are isolated in these tiny droplets. Based on measurements recorded on cells in flow, droplets containing cells of interest are charged. As the droplet stream passes through an electrostatic field, the charged droplets are deflected right or left, carrying the sorted cells.

Applications of Flow Cytometry to Cancer Biology

During 1950's and 1960's, a series of experimenis was reported (*23*) indicating that malignant cells contain more DNA per cell than is found in normal cells. These observations were made by means of absorption and fluorescence static cytophotometers measuring the DNA content of a few hundred Feulgen-stained cells on microscope slides. With the advent of flow cytometry, it became apparent that fluorescent measurements of DNA content could be made on single cells at rates of 50,000 cells per minute (*1*). Since that time a number of different fluorescent dye systems have been developed (*24–30*) to measure DNA content per cell with a flow cytometer.

The first peak of a DNA frequency distribution (Fig. 2A) is known as the G_1 peak (*31*), and the modal peak channel is a measure of the DNA content of the G_1 cells in that population. Based on static cytophotometric data (*23*), one would expect DNA frequency distributions of cells derived from tumor tissue to have a G_1 (2C) DNA peak for normal cells and a G_1 peak at a value greater than 2C for malignant cells, since they are expected to have more DNA per cell (*31*). Several studies in which zero resolution flow cytometry was used (*32*) confirm the static measurement that the DNA content of malignant cells is greater than that of normal cells. However, samples containing both normal and malignant cells can at times have G_1 cells with only the 2C DNA content. The relation of DNA content to malignancy is still under investigation.

Malignant cell types may also contain DNA which has different physicochemical properties than those found in normal cells. Thermal denaturation of DNA in situ can be studied in individual cells by staining heat-treated cells with Acridine Orange and measuring fluorescence with a flow cytofluorometer (*33*, *34*). By simultaneously measuring fluorescence at two wavelengths one can determine the amount of denatured and native DNA within a cell.

Cancer Therapy

Many therapeutic agents preferentially effect cells in one particular stage of the life cycle. For this reason it is important to know the life cycle distribution profile of both tumor and normal cells (for example, bone marrow cells) within a patient. A major goal in the treatment of patients is to achieve a state in which the bone marrow cells are in the G_1/G_0 phase of the cell cycle (*31*), and most tumor cells are in the $S + G_2 + M$ phases. With the successful achievement of this goal, it is hoped that with the proper selection of therapy normal cells could be left unharmed while cytocidal effects of therapeutic agents could be directed against tumor cells.

Several mathematical algorithms have been developed (*35*) to compute the percentage of cells in various stages of the cell cycle and other important parameters from the DNA frequency distributions. As shown in Fig. 2A, the G_1 population has a 2C DNA content (*31*). Cells that are synthesizing DNA (S phase) occupy the region in the frequency distribution between 2C and 4C. The G_2 phase cells (post-DNA synthesis and premitotic) and M phase (mitotic) cells both have the 4C amount of DNA and occupy the region of the curve under the peak on the right side of the frequency distribution.

Tobey (*36*) and Tobey and Crissman (*37*) used flow cytometry to study cell cycle perturbations caused by chemotherapeutic agents on tissue culture cells. Since that time, several additional studies have been reported (*38*) in which radiotherapeutic or chemotherapeutic agents were used. These studies provide much information concerning the effects of various agents on cells. For example, melphalan (a drug used in the treatment of breast cancer) was tested on a tissue culture human lymphoma line (T_1) (*39*). Melphalan displayed a great lethal potential and induced a block in G_2. Knowledge of cell cycle effects of therapeutic agents is of value to the clinician; however, it is no substitute for knowing the cell cycle distribution within the patient.

Several studies have been reported (*28*, *40–42*) where cell cycle parameters were obtained by using flow cytometry on bone marrow cells from leukemic patients. These studies were conducted to compare flow cytometric measurements against tritiated thymidine labeling index and to determine the usefulness of these data in the clinical management of cancer. Both Krishan *et al.* (*42*) and Barlogie *et al.* (*28*) have found that a correlation exists between the labeling index and proliferation index (percentage of cells in S or $S + G_2 + M$ as determined by flow cytometry). However, both indices (the labeling index and the proliferation index) were observed to vary from 3 to 30 percent. In healthy individuals, the proliferation index of bone marrow cells appears to remain stable at a point between 10 and 25 percent (*43*). The proliferation index has an advantage over the labeling index in that it can be obtained within 2 hours after a sample is removed from the patient. The labeling index requires a week to obtain. It is important to know the status of the bone marrow at the time therapy is administered, not a week later. Hart *et al.* (*44*) have shown that a labeling index (and presumably the proliferation index) ex-

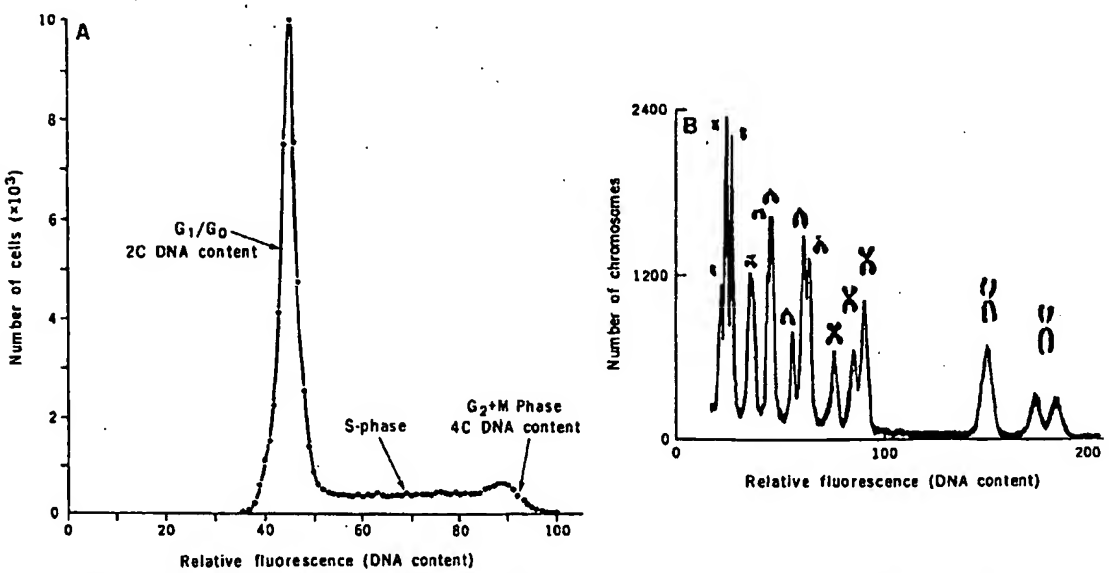

Fig. 2. (A) Ficoll/hypaque purified human bone marrow cells stained with propidium iodide (*25*). Stained cells are analyzed one at a time by means of a two-parameter cell sorter. The resulting fluorescence distribution shows a G_1/G_0 peak with 2C DNA content (peak at left of frequency distribution), a $G_2 + M$ peak with 4C DNA content (peak at right of frequency distribution), and S-phase region with DNA content of 2C to 4C (*31*). (B) Chinese hamster M3-1 karyotype as analyzed by flow cytometry. Chromosomes from Chinese hamster M3-1 line cells were isolated and stained with fluorescent dye 33258 (Hoechst). The resulting fluorescence distribution shows a number of peaks, each produced by chromosomes of a single type. The association between chromosome type and peak is illustrated. Note that the method has sufficient sensitivity to distinguish between the homologs of the No. 1 chromosome (double peak at the highest fluorescence value). The fluorescence distribution serves as a quantitative karyotype; the peak modal channels represent chromosomal type while peak areas represent the number of chromosomes of that type. [Courtesy of Lawrence Livermore Laboratory]

ceeding 9 percent is a favorable prognostic factor for successful remission of adult patients with acute leukemia when treated with chemotherapy.

The general utility of cell cycle analysis in the management of tumor patients is being investigated. Currently, the theory is that before flow cytometry can be used effectively, capability of distinguishing tumor cells from normal cells (*40*) and cycling cells from noncycling cells within a single specimen must be achieved. The cycling cells must be distinguished from noncycling cells because noncycling cells (G_0) have a 2C DNA content and are found as part of the G_1 peak, Fig. 2. This contamination of the G_1 peak with G_0 cells makes interpretation and application of cell cycle parameters difficult. Since G_0 cells respond differently to therapy than G_1 cells it is important to distinguish between these two cell stages. Several differential staining techniques have been developed by means of flow cytometry which may be capable of distinguishing cycling (G_1) from noncycling cells (G_0) (*45*). The applicability of these new methods to normal and tumor material in vivo is under study.

Even after solving the problems of noncycling cell identification and tumor cell identification in mixed populations, flow cytometric cell cycle determinations may not be immediately applicable to clinical management of cancer therapy. Problems such as drug delivery and drug permeability may vary between two patients with similar cell cycle analysis. These variables may contribute to the overall predictive outcome of therapy. Nonetheless, flow cytometry is currently providing a major new look at the delivery of anticancer agents.

Cancer Screening

Since information obtained from DNA analysis is in itself not always diagnostic of malignant cells in human gynecological specimens, other parameters have been used. Fowlkes *et al.* (*46*) and Steinkamp *et al.* (*19*) used simultaneous measurement of DNA content (propidium iodide) and protein content (fluorescein isothiocyanate), while investigators at Lawrence Livermore Laboratory (*47*) are using DNA content (chromamycin) in combination with small-angle light-scatter intensity, and more recently, 90° light-scatter intensity. Multiparameter approaches are relatively new, and whether they will prove any more useful than single parameter measurement to detect premalignant cells in gynecological samples has yet to be determined.

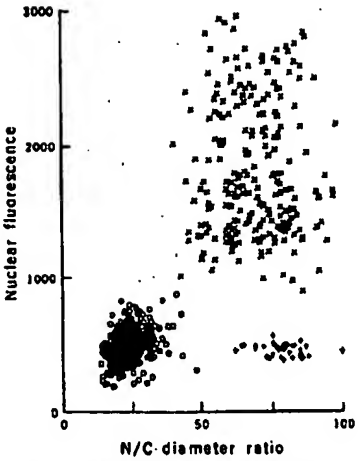

Fig. 3. Nuclear fluorescence versus nuclear to cell (N/C) diameter ratio (expressed as a percentage) for cells from human gynecological specimens. This is a composite from 16 specimens. Cell types analyzed are intermediate squamous cells (normal) (O), lymphocytes (+), and cells derived from dysplasia, metaplastic type (abnormal) (x). [Courtesy *Acta Cytol.* (*48*)]

Wheeless *et al.* (*48*) and Cambier *et al.* (*49*) have used a slit-scan technique on Acridine Orange stained cells to determine the presence of abnormal cells in both gynecological and urinary tract specimens. Nuclear fluorescence alone was documented as a sufficient descriptor for distinguishing normal from abnormal cells (see Fig. 3). Nuclear diameter (N) to cell diameter (C) ratio may be used to segment the normal cell population to detect the inadequate specimen. As shown in Fig. 3, normal intermediate squamous cells from gynecological specimens have a low nuclear fluorescence intensity and a small N/C ratio resulting in the cluster near the origin. Dysplastic (abnormal) cells have a much higher nuclear fluorescence, and lymphocytes have a high N/C ratio but a low fluorescence intensity. While slit-scan analysis provides distinction between abnormal and normal cells, the systems' ability to recognize artifact, cell overlap, and other causes of false alarms must be improved to achieve the sensitivity desired for a practical prescreening instrument.

Somatic Cell Genetics

Many genetic abnormalities involve the addition or deletion of a single chromosome. One such genetic deviation is Klinefelter's syndrome (XXY). Cells from patients with this syndrome have an additional X chromosome. Using conventional staining procedures, researchers from two laboratories (*50*) have shown that a small increase in DNA content per cell, as seen by the addition of one human X chromosome, can be detected by flow cytometry. This type of analysis can be performed only when staining and instrument variations are kept at a minimum and the G_1 peaks are extremely narrow. While additions and deletions within a single chromosome are not as yet detectable by flow cytometry, it is possible to analyze the DNA content of single chromosomes (*51*). Preparations of single chromosomes are stained with fluorescent dyes and the fluorescence measured with zero resolution flow cytometry. In Fig. 2B a sample frequency distribution histogram is displayed. The chromosome responsible is shown on top of each peak. At present these measurements are difficult to make; when the technical problems are solved, flow cytometry may provide a new and exciting method for genetic analysis of the unborn fetus.

Immunobiology

Immunological studies have made extensive use of single color fluorescence analysis and live cell sorting (*52–59*). Typically, a cell population is stained by means of a direct or an indirect fluorescence procedure (*52, 58*). As an example of indirect immunofluorescence staining and analysis, we describe a methodology for tagging surface membrane immunoglobulin (SmIg) on B cells (a subpopulation of lymphocytes). Purified lymphocytes from human peripheral blood are incubated in goat antiserum made against human SmIg. Those cells containing SmIg (B cells) react with the antiserum and have goat antibodies attached to the SmIg. The non-B cells in the population do not react with this antiserum and, therefore, do not have goat antibodies attached. To stain the B cells with a fluorescent probe, the cells (B and non-B) are then reacted with fluorescein conjugated rabbit antiserum to goat Ig. The fluorescent rabbit antibodies to goat Ig only bind to those cells with goat antibodies (B cells). Thus the B cells fluoresce green, and the non-B cells do not fluoresce.

Stained cells are then analyzed for fluorescence intensity with a zero resolution cell sorter. A typical fluorescence histogram is illustrated in Fig. 4A. This type of analysis can be performed with any surface antigen. The only limitation is availability of fluorescein conjugated antibody reagents [preferably an $F(ab')_2$

fragment; that is, a bivalent antigen binding fragment] and a sufficient fluorescence intensity per cell to permit detection (*14*). Quantitative fluorescence analysis with cell sorters has been reported for several cell surface markers. These include: Fc (crystallized fragment) receptors (*54*); KLH, or keyhole limpet hemocyanin (*52*); Θ antigen on the surface of murine T cells (*53*); Ia alloantigens on thymocytes (*55*); and SmIg (*56*). Fluorescent profiles obtained with Ig reagents (Fig. 4A) are characteristically very broad when compared with DNA frequency distributions. However, even with the broad distributions, immunobiologists can use the sorter to collect subpopulations of determined intensity to test for functional activity. For example (Fig. 4A), viable cells in channels 2 to 10 (weakly fluorescent) and channels 90 to 100 (strongly fluorescent) could be sorted into separate beakers and tested for a specific biologic function. The test may be in vivo but the end analysis would be to compare function with fluorescence intensity.

Immunofluorescent procedures are not confined to measurements of immunological parameters. These probes are used also in quantitative studies of viral antigens (*60*) and neural antigens (*61*).

Another application of flow cytometry is in the area of live-cell : dead-cell evaluations. Trypan blue, fluorescent dyes, and light-scatter intensity (*14*, *62*, *63*) have all been assessed for applicability to automated live-cell : dead-cell counting. One example of a single dye system is shown in Fig. 4B. A sample containing antibody-complement killed cells and live cells is exposed to the dye ethidium bromide (*63*), staining the dead cells and not the live cells. The sample is diluted 1 : 40 and then exposed to Zap-isoton (Coulter Diagnostics) releasing intact nuclei from live cells. The live cell nuclei now stain, but less intensely than dead cells because the stain is diluted. Fluorescence analysis reveals brightly stained dead cells (peak to the right of the histogram) and dimly stained live cells permitting quantitative evaluation of both populations. Live-dead cell analysis of this type can be carried out analyzing 2000 cells in less than 2 minutes per sample.

Blastogenic response is another common measurement made by immunobiologists which is easily accomplished by means of zero resolution cytometers (*64*). This analysis is based on the fact that nonstimulated purified lymphocytes are G_0 and G_1 cells containing 2C amounts of DNA and very little RNA. Whenever a cell is stimulated by antigen or mitogen, both RNA and DNA levels increase. The increase in either macromolecule can be quantitated by flow cytometry, permitting quantitative evaluation of the blastogenic response.

New Instrumentation Directions

Numerous new instrumentation techniques and developments are emerging which expand the feature set and subsequent information available from flow instruments. These new techniques and the adaptation of existing static techniques to flow instrumentation will have impact on second generation flow analyzers and sorters.

Light scatter. Whereas a single detector was used in early studies to record small-angle forward light scattering, in recent studies other angles, multiple detectors, and sweep techniques are used to derive additional morphological information on cells.

The use of a 32-element ring detector array for obtaining the distribution of light-scatter intensity versus angle on cells in flow is reported by Mullaney *et al.* (*65*) and Salzman *et al.* (*66*). It is too early to determine whether biological variability from cell to cell within the same cell type will mask the ability to relate signals from specific rings with cellular morphology.

A novel system by Loken *et al.* (*67*) utilizes the relative motion of a cell with respect to a fixed detector to record cellular light scatter over a continuous angle. The incident laser beam is greatly expanded, and a slit aperture is placed between the flow stream and detector. As a cell flows across the laser beam, the angle subtended by the beam and detector aperture varies providing a distribution of the scatter intensity as a function of angle from a single detector. A disadvantage of this design is the possibility of collecting scatter from more than one cell at a time because of the presence of multiple cells in the expanded laser beam.

As resolution is increased in a flow system by focusing the laser beam to a

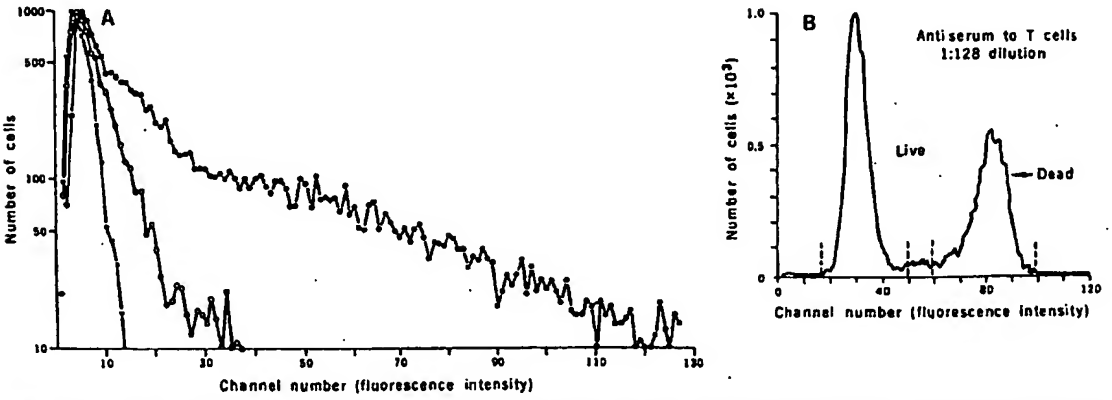

Fig. 4. (A) Fluorescence analysis of Ficoll-hypaque purified human lymphocytes from peripheral blood. Symbols: (■), unstained lymphocytes; (●), stained lymphocytes reacted with goat antiserum to human SmIg and then fluorescein conjugated rabbit antiserum to goat Ig; and (○), stained lymphocytes reacted with fluorescein conjugated rabbit antiserum to goat Ig. Fluorescence increase of frequency distribution two (○) over frequency distribution one (■) is a measure of the nonspecific sticking of the fluorescein conjugate. The B cell population can be enumerated by calculating the area between distribution two (○) and three (●) and dividing by the area under frequency distribution three (●). (B) Purified mouse T cells were exposed to complement and a limiting dilution of cytotoxic antiserum to T cells. Sample contains both live and dead cells. Proper staining of cells with Ethidium Bromide stains dead cells brightly and live cells less intensely. The fluorescence profile was obtained by means of a two-parameter sorter. The percentage of dead cells can be determined by integrating the number of cells under each peak.

narrow slit, so is the potential information increased from light scattered by a cell intersecting the line-focused laser beam. Additionally the use of slit laser radiation reduces the complex structure of a cell into a series of simpler structures making the scattered light easier to interpret. Hardy *et al.* (*68*) have determined detector configurations for use with line-focused laser beams which allow recognition of boundaries within the illuminated portion of a cell and supply information on overall cell orientation. Sharpless *et al.* (*69*) report similar information and refractive index determination on transparent test particles. These investigations indicate that light scattered from objects irradiated with a line-focused laser beam carries useful information on the structure of cells which can be extracted in flow with proper detector design.

Multiple sources. Several investigators have utilized multiple sources to further increase the feature set available in flow. The sources may be combined in a single beam or displaced from each other providing sequential illumination.

Stöhr (*70*) combined the output of an argon-ion laser (488 nm) with that of a helium-cadmium (441 nm) laser for simultaneous excitation of two differently absorbing fluorescent agents bound in a cell. Having the advantage of simultaneous measurement of two different emission signals, the technique is limited by color overlapping and energy transfer.

Sequential laser beams were first used in flow by Hulett *et al.* (*10*) to record light scatter followed by fluorescence on cells. Current systems use up to five measurement stations (*71*). An instrument incorporating three laser stations each capable of recording up to five optical measurements per cell is described by Shapiro *et al.* (*72*). The stations are spaced 100 μm apart, use three different wavelengths for illumination, involve cell rates of over 1000 cells per second, and are consistent with seven-parameter data recording. A requirement on this type of instrument is a large memory for storage of correlated multiparameter data. Correlation of data between detector stations is assisted by monitoring the time interval between cell arrival at the first and third stations and using this information to adjust detector timing.

Imaging in flow. Techniques have been developed to provide images of cells in flow for study of cell orientation and dynamics, and correlation of measured features with cell type. Kachel (*73*) equipped a microscope with a flow-measuring chamber and used a nanosecond flash lamp to record images of red blood cells for study. Kay *et al.* (*74*) obtained multiple images of squamous cells in flow with a laser stroboscopic technique. This technique provides either dark-field or phase-contrast multiple images of a cell on a single frame of film from which cell orientation, velocity, and acceleration may be obtained.

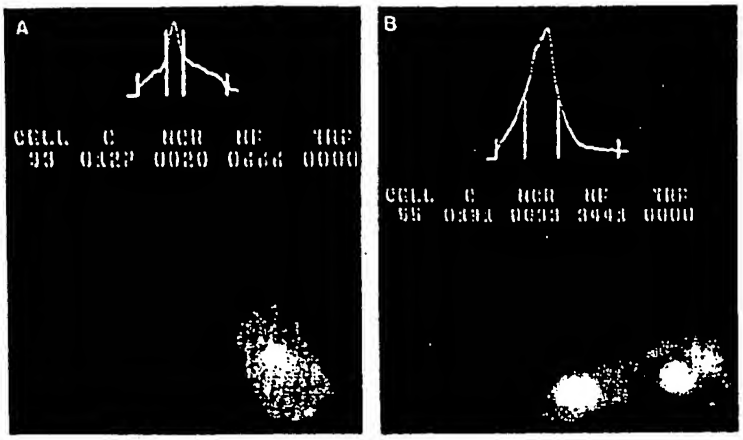

Fig. 5. Vidicon images of cell fluorescence recorded on a swept slit-image flow correlation system. For each photograph: 540 nm slit-scan contour (top) with computer-determined cell and nuclear boundaries (vertical lines), contour analysis data, and vidicon image of cell (bottom). (A) Intermediate squamous cell. (B) Squamous cells overlapping along direction of laser excitation slit resulting in misclassification as abnormal cell. [Courtesy of *J. Histochem. Cytochem.* (*75*)]

A "swept slit image" flow correlation system developed at the University of Rochester (*75*) yields two-dimensional vidicon images of cell fluorescence with a resolution of approximately 2 μm (Fig. 5). As cells flow through a slit of laser excitation illumination, a scanning mirror is triggered to sweep the image of the slit-scan region across a silicon intensifier target vidicon camera tube at a rate related to the cells' flow velocity. A fluorescence image of each cell is swept on the tube. The video image is stored and used for correlation with the cells' slit-scan fluorescence contour which is recorded at the same time.

These imaging systems are important both for the information they yield and because they move us closer to increasing the feature set available to flow analysis by also providing the potential for image analysis in flow.

Cell orientation. Effects of cell orientation and dynamics on measurements recorded in flow are attracting increased interest. Cell orientation may greatly affect slit-scan contours and resulting data in flow systems having line focused excitation illumination (*18*, *76*). Recent studies have shown that even in some zero resolution flow systems, the orientation of nonspherically symmetric cells (for example, sperm) may affect fluorescence measurements (*77*, *78*). In addition, orientation will affect light-scatter measurements (*68*, *69*). Approaches to this problem are twofold: attempts are made to control cell orientation (*76*, *79*) and to design systems whose measurements are unaffected by cell orientation (*78*, *80*).

Other. Simultaneous measurement of the linearly polarized fluorescence emission vectors oriented parallel and perpendicular to the polarized laser excitation may be accomplished on a cell by cell basis in flow (*81*). With the use of suitable fluorescent probes, these measurements provide quantitation of intracellular microviscosity or viscosity of cellular membrane lipids.

The determination of "opacity" is a flow measurement with interesting potential which should be surfacing from research laboratories in the near future (*82*). Opacity is defined as the a-c impedance generated by a particle in an orifice (measured at high frequencies) divided by the standard d-c Coulter electronic cell "volume."

Also exciting is the potential use of flow cytometers and sorters as components of larger systems. Hybrid systems are envisioned in which flow cytometers and sorters will be used as first-stage processors to identify and sort out subpopulations of cells, chromosomes, and bacteria, for example, for subsequent processing and analysis. Cancer screening or cell classification instruments, for example, might incorporate low-resolution flow analyzers to identify a cellular subpopulation containing abnormal cells which would be sorted out for higher resolution two-dimensional image analysis to determine

precisely which cells are abnormal (*75*). These cells might in turn be automatically restained and rescanned at even higher resolution for cell classification and disease prognosis.

New Applications

New flow applications may be classifed as those which utilize new instrumental procedures and those which use new methodological approaches. One new methodological approach involves looking at the distribution of binding sites on the surface of a cell (*83*). Spherical cells, stained uniformly on the surface with a light-absorbing dye, scatter light between 2° and 12° off the main laser beam with a given intensity. If the stained sites are allowed to migrate and the dye collects at one pole of the cell, the light-scatter intensity will increase. The usefulness of flow cytometry in studying the distribution of surface receptors is now being evaluated.

The cytometers discussed in this article require the addition of fluorescent markers to the sample in order to visualize biochemical events in cells. A new system has recently been developed which makes use of small unilamellar lipid vesicles (liposomes) containing high concentrations of fluorescent dyes (*84*). These liposomes are used as vehicles for introducing fluorescent membrane-impermeable substances into cells.

Methods for increasing fluorescence intensity or making possible the detection of cellular components that are found in low concentration have long been a subject of interest in flow cytometry. One novel procedure is to attach specific antibodies to small microspheres (*85*). As an example, goat antiserum to human Ig is placed on 0.8 μm spheres. These functional microspheres are now placed in contact with purified human lymphocytes. Those cells with surface Ig will have many microspheres attached. As cells are passed through the cell sorter, tagged cells can be distinguished from nontagged cells on the basis of light-scatter intensity. It is also possible to fill the microspheres with fluorescent dye and detect the tagged cells on the basis of differential fluorescence intensity.

Based on a concept by Fulwyler (*86*), Horan *et al.* (*87*) developed a methodology that permits quantitative evaluation of autoimmune dysfunction. As a definition of autoimmune dysfunction let us assume that antigens A, B, and C are present in normal individuals, but that antibody response is generated against these antigens only in diseased individuals. We can measure the intensity of the antibody response against an antigen by using the following technique: Antigen A is bound to 5-μm uniform polystyrene microspheres, antigen B to 11-μm polystyrene microspheres, and antigen C to 14-μm polystyrene microspheres. These spheres are very uniform and do not overlap in their size distributions. To check a serum for antibodies against these antigens, one places all three sizes of functional microspheres into the test serum. After the microspheres are washed free of unbound serum, the spheres are reacted with fluorescein conjugated antiserum to human Ig to detect the presence of human antibodies. If the patient has antibody against antigen A, the microspheres exposed to patient serum will fluoresce brighter than microspheres exposed to normal serum. These microspheres are then analyzed by means of flow cytometry, Fig. 6. The size classes (and therefore the antigen) can be identified by light-scatter intensity. The concentration of antibody in the serum specific for antigen A, B, or C is measured by fluorescence intensity.

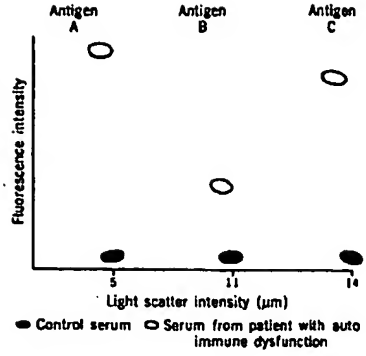

Fig. 6. Idealized histogram for the detection of autoantibodies in serum of a patient with autoimmune dysfunction. Three sizes of plastic microspheres each have bound a different antigen. If a patient has antibodies against antigen A, the fluorescence intensity will be greater than that found for control serums.

Conclusion

Quantitative multiparameter flow analysis and sorting of cells is emerging as one of the important new technologies in biological and biomedical research and clinical medicine. The ability to obtain measurements on single cells at very high rates and to sort these cells for further analysis has enabled biological researchers to ask questions that heretofore were not approachable because analytical methods did not exist to provide the needed data. This article describes the technical aspects of flow cytometric instrumentation and its applications, including some of the more innovative new approaches.

The interdisciplinary approach required in flow cytometry has brought together engineers, biophysicists, biologists, and clinicians for the development and application of this new technology. By the end of the next decade, flow cytometry may be as important in research and clinical medicine as the scintillation counter or the Autoanalyzer (Technicon Instruments, Inc.).

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